Human Molecular Genetics, 2007, Vol. 16, No. 16 ***1921–1930***
doi:10.1093/hmg/ddm139
Advance Access published on June 12, 2007

GNAS transcripts in skeletal progenitors: evidence for random asymmetric allelic expression of Gsα

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Received March 23, 2007; Revised May 9, 2007; Accepted May 13, 2007

Activating mutations of the Gsα gene, encoded by the guanine nucleotide-binding protein, alpha stimulating (*GNAS*) locus located on chromosome 20q13, underlie different clinical phenotypes characterized by skeletal lesions [fibrous dysplasia (FD) of bone], extraskeletal diseases (mainly endocrine hyperfunction and skin hyperpigmentation) and variable combinations thereof [the McCune–Albright syndrome (MAS)]. This clinical heterogeneity is commonly assumed to reflect the post-zygotic origin of the mutation. However, the pattern of imprinting of the Gsα gene in some human post-natal tissues suggests that parental-dependent epigenetic mechanisms may also play a role in the phenotypic effect of the mutated *GNAS* genotype. FD lesions are generated by mutated clonogenic osteoprogenitors that reside, along with their normal counterparts, in FD bone marrow stroma. We analyzed the allelic expression pattern of Gsα and other *GNAS* alternative transcripts in the progeny of normal and mutated clonogenic stromal cells isolated *in vitro* from a series of informative FD/MAS patients. We report here for the first time that the two Gsα alleles are unequally expressed in both normal and FD-mutated stromal clones. However, in contrast to imprinting, the ratio of Gsα allelic expression is randomly established in different clones from the same patient. This result suggests that a parental-independent modulation of Gsα expression occurs in clonogenic osteoprogenitor cells and, at the single cell level, may impact on the severity of an FD lesion. Furthermore, we show that normal and mutated clonogenic stromal cells express *GNAS* alternative transcripts other than the common Gsα, some of which may be relevant to the development of FD.

INTRODUCTION

Fibrous dysplasia (FD) is a skeletal disorder caused by post-zygotic activating mutations of the Gsα gene (usually R201C, R201H, and occasionally R201S and R201G) (1–5), which is encoded at the guanine nucleotide-binding protein, alpha stimulating (*GNAS*) locus on chromosome 20q13. Although all FD patients share mutation at R201, their clinical phenotype is remarkably variable, depending on the degree of somatic mosaicism. FD may occur as an isolated disorder (6,7) or as a part of more complex disease phenotypes, which include hyperfunctioning endocrinopathy(ies) and skin hyperpigmentation in the McCune–Albright syndrome (MAS) (8–11), and muscular myxoma in the setting of FD (Mazabraud's syndrome) (12,13). The clinical expression of FD itself is heterogeneous, ranging from monostotic and/or asymptomatic lesions to polyostotic and severely crippling disease (14). While the extent of the disease is highly variable from patient to patient, FD lesions are commonly characterized by replacement of normal bone and marrow with structurally abnormal bone and a fibrotic marrow, as a consequence of constitutively active Gsα and overproduction of cAMP (14).

The Gsα gene belongs to the *GNAS* locus that encodes multiple alternative transcripts, including Gsα (15), the extra-large

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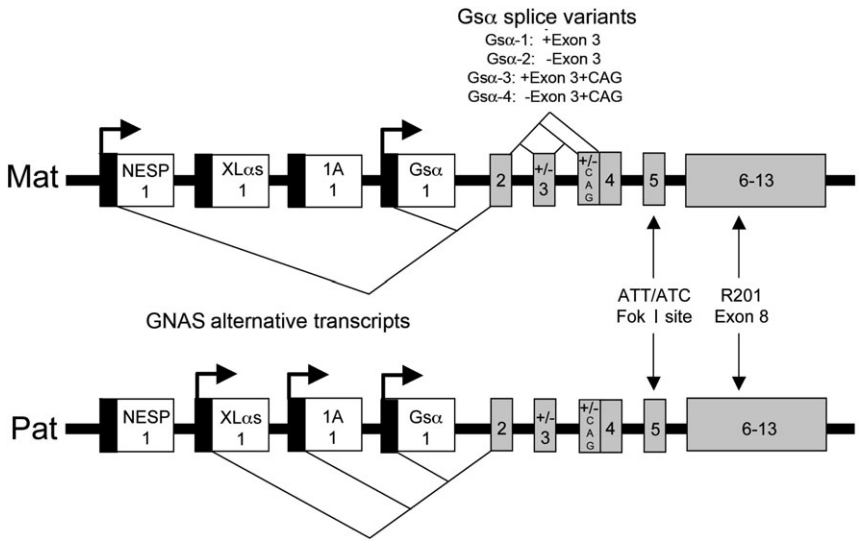

Figure 1. Scheme of *GNAS* chromosomal region showing promoters (black boxes) and 1st exons specific to each transcript (NESP55, XLαs, 1A and Gsα) as white boxes and exons common to all *GNAS* transcripts as grey boxes (exons 2–13). NESP55 is reported to be expressed from the maternal allele (Mat), whereas XLαs and 1A are reported to be expressed from the paternal allele (Pat). Gsα is reported to be expressed from both alleles in most normal tissues, and four different splice variants have been report: Gsα-1 (long, with exon 3), Gsα-2 (short, without exon 3), Gsα-3 (long form with CAG immediately upstream of exon 4) and Gsα-4 (short form with CAG). The ATT/ATC polymorphism, the site of Fok I restriction, is in exon 5, and the R201 FD mutation site is in exon 8.

form, XLαs (16,17), the chromogranin-like protein, NESP55 (16) and the untranslated transcript, 1A [also known as A/B (18–20)]. This complex pattern of the alternative transcript expression results from the presence of multiple promoters and first exons that splice onto a common downstream coding sequence (exons 2–13) (Fig. 1). In addition, transcription of the Gsα gene generally gives rise to four different Gsα splice variants: Gsα-1 (long, containing exon 3), Gsα-2 (short, without exon 3), Gsα-3 [long with an additional CAG (Serine) preceding exon 4] and Gsα-4 (short with the additional CAG). Other Gsα mRNA species have also been reported, although at low frequencies (15).

The clinical heterogeneity of FD patients is widely assumed to be a result of the somatic nature of Gsα-activating mutations and mosaics arising from varying combinations of normal and mutated cells in skeletal and extraskeletal tissues (21). However, concomitant epigenetic and/or microenvironmental factors may also play a role. Imprinting is an epigenetic phenomenon that leads to parental-dependent monoallelic patterns of gene transcription, and is usually established through the methylation of the promoter of the inactive allele (22–24). In the mouse and in humans, the *GNAS* locus has a complex status of imprinting (25). NESP55, and XLαs and 1A have opposite patterns of allelic transcription, with the NESP55 promoter being methylated on the paternal allele and the Lαs and IA promoters being methylated on the maternal allele (16,17,26). The Gsα promoter has been reported to be unmethylated on both alleles in most human adult tissues (16,17,26) and its transcription is biallelic in human fetal tissues (27). However, recent studies have reported prevalent maternal expression of the Gsα gene in some human postnatal tissues such as the pituitary gland (28), the thyroid and gonads (29).

We have previously shown that fibrous dysplastic bone is produced by the differentiated progeny of mutated skeletal stem cells (30,31). Either normal or Gsα-mutated clonogenic skeletal stem/progenitor cells may be isolated and expanded *ex vivo* from the fibrotic marrow of FD lesions (31,32). In this study, we investigated the allelic expression pattern of Gsα in clonal populations of stromal cells originating from individual colony forming unit-fibroblasts (CFU-Fs) grown *in vitro* from FD/MAS patients and separated according to their genotype. Furthermore, we analyzed the expression and allelic transcription pattern of other *GNAS* alternate transcripts in the same cell types.

RESULTS

Identification of FD patients and normal donors heterozygous for the ATT/ATC polymorphic site in the *GNAS* gene

To determine the allelic expression of *GNAS* transcripts, both in normal and mutated cells, we chose heterozygosity for the ATT/ATC polymorphic site in exon 5, which is present in all of the alternative transcripts encoded by the *GNAS* locus as a suitable marker (27). Genomic DNA was extracted from fresh tissue samples or marrow stromal cell cultures from 17 FD patients and 12 normal donors (donor characteristics are listed in Table 1). A 429 bp target sequence of the *GNAS* gene, including the ATT/ATC polymorphic site in exon 5, was amplified by polymerase chain reaction (PCR) and digested with Fok I, which specifically cleaves only the amplification product with the ATC sequence. Eight ATT/ATT individuals (five FD patients and three normal donors) were identified by the absence of digestion of the PCR products

Table 1. Patients characteristics

Subjects	Clinical data	Sex	Age range (years)	R201 mutation	ATT/ATC
17 FD	15 MAS	9 F	8–59	12 R201C	8
	1 PFD	8 M		2 R201H	
	1 MFD			3 NA	
12 N		6 F	15–45		6
		6 M			

FD, fibrous dysplasia patient; NA, not assessed; MAS, McCune–Albright syndrome; PFD, polyostotic fibrous dysplasia; MFD, monostotic fibrous dysplasia.

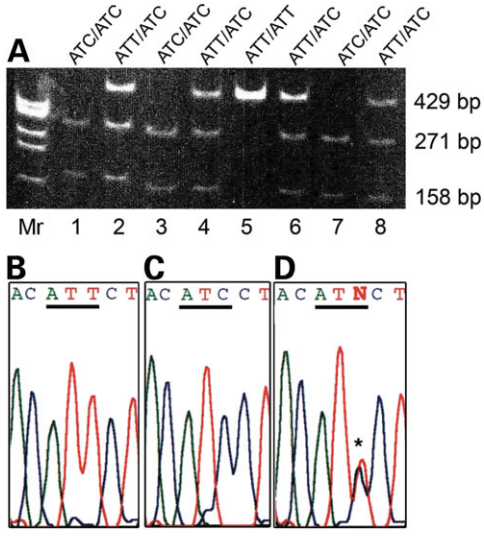

Figure 2. (**A**) Analysis of ATT/ATC polymorphism at the exon 5 of *GNAS*. A 429 bp target sequence was amplified by PCR and digested with Fok I. The presence of undigested PCR product (429 bp) only identified ATT/ATT patients (lane 5, **B**). ATC/ATC patients were recognized by complete digestion of the amplicon to 271 and 158 bp fragments (lanes 1, 3 and 7, **C**), ATT/ATC patients were identified by the presence of undigested PCR produce and the two digested fragments (lanes 2, 4, 6 and 8, **D**). DNA sequencing confirmed the ATT/ATT (B), ATC/ATC (C) and ATT/ATC [ATN polymorphism noted by an asterisk in (D)] genotype in each patient.

(representative sample in Fig. 2A, lane 5). Complete cleavage of the amplicons into two fragments of 271 and 158 bp, respectively, enabled us to identify seven ATC/ATC individuals (four FD patients and three normal donors) (representative samples in Fig. 2A, lanes 1, 3, 7). The presence of both uncleaved and digested amplification products identified 14 ATT/ATC individuals (eight FD patients and six normal donors) (representative samples in Fig. 2A, lanes 2, 4, 6, 8). The results of restriction analyses were confirmed by DNA sequencing of all samples (representative samples in Fig. 2B–D).

Cloning of normal and FD-mutated stromal CFU-Fs

Since FD lesions are mosaics that include both normal and FD-mutated (R201H, R201C) stromal cells (31), individual stromal stem/progenitor cells (CFU-Fs) of either genotype were isolated by cell cloning as described previously (33). Clonal cultures were established starting from single-cell suspensions prepared from marrow samples of 4 FD patients with both the ATT and ATC alleles [patients (Pts) 4, 8, 10, 13] and 23 clones originating from single CFU-Fs were successfully expanded. Each clone was analyzed by PCR amplification and sequencing of a target fragment of genomic DNA including the R201 mutation site in exon 8. These methods identified 16 clones with the normal genotype and 7 with the FD-mutated genotype. A non-clonal stromal strain, including both normal and R201C FD-mutated stromal cells, was available from another ATT/ATC polymorphic patient, Pt 17. A summary of FD cell samples available for the study is reported in Table 2.

Table 2. FD BMSC populations

FD patient	Gender, age (years), diagnosis	BMSC cultures		
		Normal clones	Mutated clones	Non-clonal
4	F, 37, MAS	1	1 (R201C)	
8	F, 22, MAS	2	1 (R201C)	
10	M, 11, MAS	12	3 (R201H)	
13	M, 11, MAS	1	2 (R201C)	
17	M, 17, PFD			R201C

MAS, McCune–Albright syndrome; PFD, polyostotic fibrous dysplasia.

Reverse transcriptase–PCR amplification of Gsα transcripts

Since the *GNAS* locus generates multiple alternative transcripts that are driven by different promoters and distinguished from one another by their corresponding first exons, a forward primer specific for the 5′ region of the Gsα transcript and a reverse primer designed from a sequence in exon 6 common to all the Gsα splice variants were used to perform reverse transcriptase–PCR (RT–PCR). Therefore, the target cDNA sequence chosen for the amplification included exon 1 (specific for Gsα) and the ATT/ATC polymorphic site in exon 5, with or without exon 3 [the long and short splice variants, respectively, of Gsα transcripts (15,34)]. RT–PCR was performed on RNA extracted from individual normal and FD-mutated clonal strains of stromal cells, as well as from non-clonal stromal cell culture strains from FD and normal donors (all from donors with the ATT/ATC polymorphism), and amplified products were resolved by electrophoresis. Two transcripts, corresponding to the long- and short-Gsα splice variants (with and without exon 3, Gsα-1 and Gsα-2, respectively), were expressed in all samples (upper two bands, uncleaved by Fok I, shown in Fig. 3A). It is not possible to distinguish the long and short form with or without the additional CAG (Gsα-3 and Gsα-4) by gel electrophoresis. Therefore, the product migrating at 459 bp may contain both Gsα-1 and Gsα-3, and the product migrating at 414 bp may contain both Gsα-2 and Gsα-4.

Imprinting of Gsα in normal and FD-mutated marrow stromal cells

The presence of the ATT/ATC polymorphism in exon 5 renders the amplification product sensitive to the action of

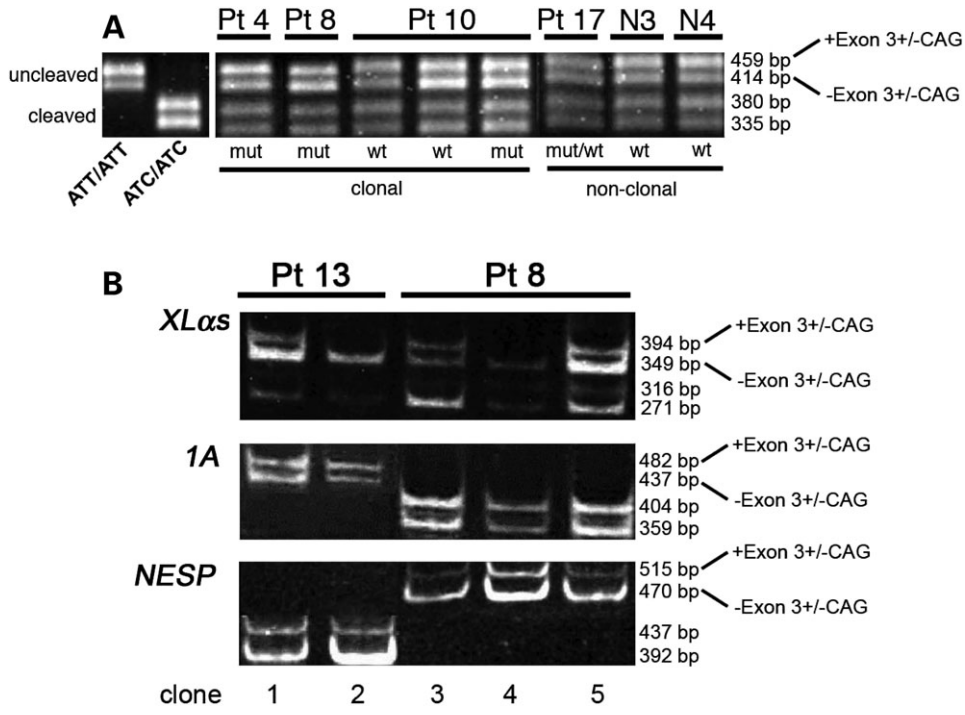

Figure 3. (**A**) Amplification of Gsα cDNA from polymorphic patients followed by digestion with Fok I. Multiple bands were detected in all eight samples corresponding to both the undigested ATT cDNA (459 and 414 bp, + and − exon 3, respectively) and cleaved ATC cDNA (380 and 335 bp, + and − exon 3, respectively, and a common 79 bp cleavage product (not shown). ATT/ATT cDNA and ATC/ATC cDNA were used as controls (left). (**B**) Expression and allelic origin of XLαs, 1A and NESP in normal and FD-mutated bone marrow stromal cells. Five representative clones from two FD patients, two with a normal genotype (lanes 1 and 3) and three bearing an FD-mutation (lanes 2, 4 and 5). cDNAs were amplified using transcript-specific forward primers and the common reverse primer, and then digested with Fok I. Restriction analysis of XLαs RT–PCR samples revealed multiple bands, corresponding to both the uncleaved (ATT) and the cleaved (ATC) cDNA. The monoallelic origin of 1A and NESP55 transcripts was demonstrated by either a complete lack of cleavage, or complete cleavage, and showed an opposite pattern of allelic expression in the same patient.

the restriction enzyme, Fok I, which can then be used to determine the allelic expression pattern. RT–PCR amplification products for Gsα were then digested to completion with Fok I and analyzed by 2% agarose gel electrophoresis. Fok I-digested (380 and 335 bp) and undigested Gsα PCR products (459 and 414 bp) were identified in all polymorphic samples, indicating that Gsα transcripts originated from both the ATT and the ATC alleles in both normal and FD-mutated cells (Fig. 3A). Densitometric analysis of cDNA electrophoresis following Fok I digestion indicated that each allelic product ranged between 40 and 60% of total cDNA (data not shown).

The alternative splicing of exon 3 in Gsα transcripts does not allow for direct sequencing of the cDNA amplification products to verify the polymorphism in exon 5. Consequently, to separately amplify and analyze the long (+ exon 3) and short (− exon 3) RT–PCR fragments, Gsα cDNAs obtained from two normal and two mutated stromal cell clones were cloned into the PCR–TOPO TA plasmid vector. Sequencing of 12 plasmid clones per sample (48 in total) demonstrated that: (i) both ATT and ATC PCR products of the polymorphic site in exon 5 were present in each original sample (Table 3); (ii) sequences included the Gsα-1 and Gsα-2 splice variants (+/− exon 3, 15/48 and 11/48, respectively) and (iii) the sequences also included the Gsα-3 and Gsα-4 splice variants (+/− CAG, 6/48 and 16/48, respectively). In all stromal cell clones examined, the four splice variants of Gsα were transcribed from both alleles.

Table 3. Summary of plasmid sequencing

Patient	Clone genotype	Exon 5 ATT/ATC
4	R201	7/5
4	R201C	6/6
8	R201C	5/7
13	R201	7/5

Expression and imprinting of other *GNAS* transcripts in normal and FD-mutated marrow stromal cells

Expression of XLαs, 1A and NESP55 in normal and FD stromal cells grown *in vitro* was assessed by RT–PCR using different forward primers specifically recognizing the first exon of each transcript and the reverse primer from exon 6, common to all alternative transcripts. XLαs and 1A cDNAs were amplified in all cultures available for the study (normal and FD-mutated clones from FD tissue, non-clonal FD cells and non-clonal cells derived from normal donors, examples shown in Fig. 3B). In contrast, transcription of NESP55 was detected in only a subset of cell populations (examples shown in Fig. 3B), including 4 FD-mutated clones, 13 normal clones, the FD non-clonal strain, and 2 non-clonal populations from normal donors.

Digestion of XLαs, 1A and NESP55 PCR products with Fok I showed that 1A and NESP transcripts originated only from one allele and had an opposite pattern of allelic transcription in each individual patient (Fig. 3B). Interestingly, Fok I digestion of the XLαs amplification product showed the presence of both the uncleaved ATT cDNA and the digested ATC cDNA, thus suggesting that in our bone marrow stromal cells samples, this *GNAS* product was transcribed from both alleles (Fig. 3B).

Quantitative analysis of Gsα allele expression in normal and FD-mutated marrow stromal cells

Owing to the relative insensitivity of densitometric measurements, and to better assess the contribution of each allele to the total Gsα cDNA in our samples, we used a quantitative PCR (q-PCR) approach based on the allele discrimination method (35). Because exon 5 is included in all of the different Gsα splice variants, q-PCR analysis was performed on the target Gsα cDNA previously amplified by standard RT–PCR, which cannot discriminate between the ATT and ATC alleles as a result of single base transition distant to the primer binding site. q-PCR was performed using two FAM-labeled probes that specifically recognize the ATT or the ATC sequence in exon 5 in the amplification products. q-PCR confirmed that expression of Gsα was biallelic in all samples analyzed, including 6 FD-mutated clones, 15 normal clones, 1 non-clonal strain from an FD patient and 3 non-clonal strains from normal donors (Fig. 4A). Compared with densitometric analysis, however, q-PCR revealed a greater variability in the relative allelic expression level of Gsα across our sample series. The proportion of transcript originating from each Gsα allele was found to be in a range between 20 and 80% by q-PCR (Fig. 4A), indicating that one allele could be preferentially, although not exclusively, expressed compared with the other allele. Interestingly, the analysis of different clonal samples obtained from a single donor revealed that either of the two alleles could preferentially be expressed in different cell clones (Fig. 4A, Pt 10). Repeat analyses of the same clones resulted in the same pattern, thus indicating that the relative ratio of Gsα allelic expression was independent of the parental origin of the allele and randomly established at the single-cell level. No relationship could be established between the genotype (normal or FD-mutated) and preferential transcription from either the ATT or the ATC allele in different clones from the same FD lesion (Fig. 4A, Pt 10).

The random pattern of Gsα allelic expression was in sharp contrast to the results of q-PCR analysis performed on NESP55 cDNAs from three FD patients and two normal donors (Fig. 4B). After RT–PCR amplification using the NESP55-specific forward primer, cDNAs from two FD-mutated clones (Pts 4 and 10), two wild-type clones (Pts 10 and 8) and two normal non-clonal strains [normal patients (N) 3, 5] were analyzed by q-PCR using the same primer set and *Taq*-Man probes used for Gsα cDNA. As expected based on the result of restriction enzyme analysis, >95% of the NESP55 cDNA in each sample showed the ATT or ATC sequence in exon 5. Furthermore, the pattern of allelic expression was concordant in different clones from the same patient (Fig. 4B, Pt 10). This result confirmed the monoallelic origin of the NESP55 transcript in bone marrow stromal cells and supported the validity of our PCR approach for quantitative analysis of Gsα allelic expression.

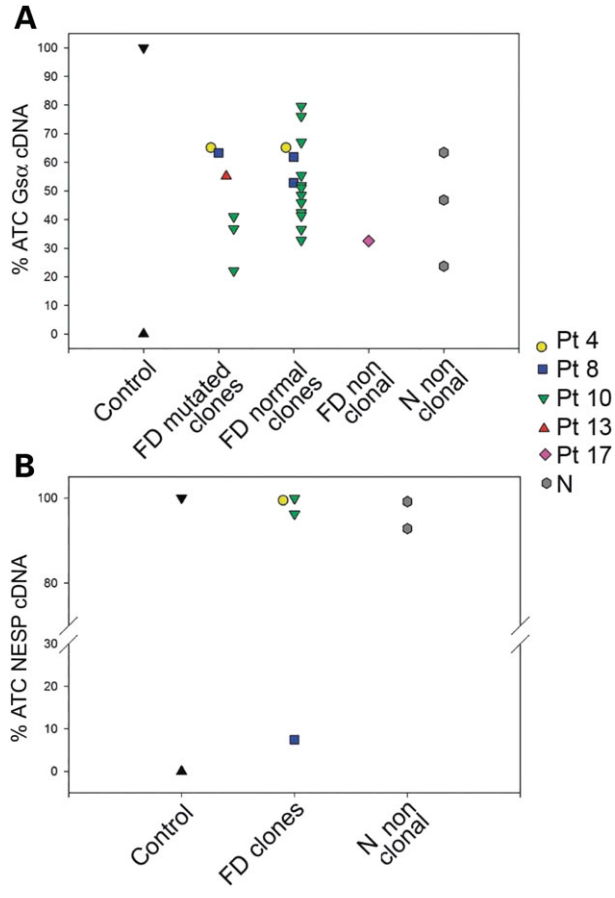

Figure 4. (**A**) Allele discrimination analysis by q-PCR of Gsα transcript in normal and FD stromal cells. A wider range of variability in the relative proportion of ATT and ATC Gsα cDNAs was detected compared with densitometric analysis (data not shown). The prevalent cDNA type was not concordant in different clones from the same patients (Pt 10). (**B**) Allele discrimination analysis by q-PCR of NESP55 transcripts in normal and FD-mutated stromal cells. The analysis of NESP55 cDNAs showed that one of the two allelic transcripts contributed to more than 95% of the total cDNA and the pattern of allele expression in different clones from the same patients was concordant (Pt 10).

DISCUSSION

We have shown that in normal and FD-mutated stem/progenitor cells, the allelic pattern of expression of the Gsα transcripts is not imprinted. We have also shown for the first time that a random asymmetry of Gsα allelic expression occurs among different clones of the same cell type. At the single-cell level, this may play a role in the phenotypic variability in individual FD lesions.

The allelic origin of the Gsα transcript in human normal and FD bone was previously investigated by Mantovani *et al.* (36,37). They reported similar levels of expression of the

two Gsα alleles in normal fetal and post-natal bone, and FD bone. Although these data seem to rule out a parental-dependent regulation of Gsα expression in bone as a tissue, they do not allow definitive conclusions on the allelic pattern of transcription of the gene in individual osteogenic cells. A slight alteration in the pattern of expression from the maternal or paternal allele, undetectable at the gross tissue level, may be possible in the osteogenic lineage, or in the specific compartments therein. This could have an impact on the local nature or 'severity' of areas within individual FD lesions in the absence of clearly segregated parental-dependent phenotypes.

The severity of disease in individual FD patients is established by the pattern of migration and survival of the progeny of the mutated cell during embryonic development, such that FD patients display highly variable degrees of somatic mosaicsim, ranging from monostotic to virtually panostotic disease. 'Severity' of an individual FD lesion relates to the degree that a number of phenotypic features that we have previously described are noted (14). These include (i) predominance of woven bone versus lamellar bone, (ii) the complete replacement of normal marrow with a fibrotic tissue, (iii) the level of cell retraction of stromal cells and of osteoblastic cells leading to formation of Sharpey's fibers, (iv) the degree of osteomalacia and (v) the presence of osteoclasts induced by the overproduction of IL-6. These features are a direct consequence of overproduction of cAMP by FD-mutated cells. Consequently, asymmetric expression from either the normal or FD-mutated allele would impact on the local level of cAMP and subsequent development of abnormalities. Attempts to address this issue may only be revealed by the analysis of the allelic pattern of Gsα expression performed on individual cells. In FD, a cell-based approach is further called for by at least two pathogenetic aspects of the disease. First, the development of FD lesions critically depends on the adverse effect of activating Gsα mutations on single skeletal stem/progenitors cells (31,38), the imprinting status of which has never been addressed. Second, the FD tissue is a mosaic composed of normal and mutated skeletal stem/progenitor cells (31). Any potential change in the relative ratio of Gsα allelic expression brought about by the activating R201 mutation, as previously reported in other tissues (28), would remain undetected unless separate analyses of normal and FD-mutated cells are performed.

Skeletal stem cells and progenitors are included in the subset of bone marrow stromal cells isolated *in vitro* as clonogenic cells (CFU-Fs) (39). They are further distinguished by their ability to expand in number through multiple passages. Thus, our data represent the allelic expression pattern of skeletal stem cells and progenitors, although it is not possible to assign our clonal strains as being derived from one or the other because of a lack of specific markers that can distinguish between them. Each clonal strain that we analyzed was the progeny of normal or R201-mutated CFU-Fs isolated from FD patients, and Gsα was detected in all. Consequently, these data suggest a role for the Gsα/cAMP signaling pathway not only in the skeletal stem cell physiology, but also in the very early differentiative stages of all skeletal phenotypes and totally agrees with the complete derangement of the bone microenvironment observed in FD (40). We also report here the first evidence that bone marrow stromal cells express all the four splice variants of Gsα (with and without exon 3, with and without CAG) (15,34).

In contrast to the previous studies, largely based on densitometric analysis, we used a very sensitive q-PCR allele-specific amplification in order to assess the relative ratio of Gsα allelic expression (35). This method has revealed that different expression levels of the two Gsα alleles are established in the majority of FD stromal clones bearing either the normal or the R201-mutated genotype. This strongly suggests that allelic expression is asymmetrically regulated in clonogenic bone marrow stromal cells. Allelic expression imbalance has been associated with expression of polymorphisms in individuals that are heterozygous for the polymorphism (41). It is thought that certain polymorphisms result in changes in transcriptional initiation and/or in mRNA stability (42–45). However, allelic expression imbalance for a particular gene is an inherited trait (41,46). Since the allelic origin of the prevalent Gsα cDNA was unequal in different clones from a single patient (Pt 10), the regulation of Gsα expression in the bone marrow stroma seems to be related to mechanism(s) other than classical imprinting and allelic expression imbalance. Instead, Gsα appears to exhibit random allelic asymmetry. This may be related to replication timing, where one allele is replicated early and the other late in the S phase of the cell cycle as has been noted for the B cell receptor loci (47). It is thought that this asynchronous replication is randomly established during development, clonally inherited, and leads to allelic exclusion (47). However, this process leads to the complete exclusion of one allele (48), whereas normal and FD-mutated stromal cells exhibit a great deal of variation, which may relate to their undifferentiated state. Lastly, the apparent asymmetric allelic expression does not appear to be because of instability of FD-mutated mRNA since varying levels were also detected in clones with normal genotype.

Although the mechanism(s) and the physiological meaning of the random allelic asymmetry of Gsα expression in human osteogenic progenitors must be further elucidated, it is conceivable that this phenomenon might act as a modifier of the FD phenotype. On the basis of this observation, in fact, it may be hypothesized that the development and severity of FD lesions are affected by not only the number of FD-mutated stem cells, but also by the fraction of stem cells in which the mutated allele is more highly expressed than the normal allele.

We also report here the first evidence of the expression of other *GNAS*-alternative transcripts (XLαs, 1A and NESP55) in normal and FD bone marrow stromal cells. XLαs is an extra large G protein (49) with a neuroendocrine-specific tissue distribution (50) that is thought to be involved in suckling and energy metabolism (51). Although it is difficult to hypothesize a specific role for XLαs in osteogenic cells at this time, the expression of this GNAS-alternative transcript could be particularly relevant in the context of FD. XLαs protein maintains the same structural domains as Gsα (49,50); its activity is comparable to Gsα activity in stimulating adenylyl cyclase in transfected cells (52). Furthermore, inactivating *GNAS* mutations that impair the Gsα function also reduce XLαs activity (53). These data suggest that

XLαs may also play a role in the cAMP-mediated signaling pathway and that the R201-mutated protein may contribute to its abnormal activation in FD. Interestingly, our results indicate biallelic origin of the XLαs transcript both in FD-mutated and in normal stromal clones, although there appeared to be some variability in the amount of Fok I cleavage (Fig. 3B), suggesting an asymmetric pattern of expression. This finding is in contrast with other data reporting the paternally restricted expression of XLαs in various differentiated tissue (17), and may be possibly explained by the stem/progenitor (undifferentiated) nature of our cells. Although further studies are required to confirm this observation, the lack of imprinting of XLαs in clonogenic marrow stromal cells is particularly important in the context of FD in that it implies that the expression of the mutated XLαs protein in the bone marrow is independent of the parental origin of the mutated allele.

NESP55 is a member of the chromogranin family expressed in the adrenal medulla, pituitary gland, brain, peripheral nerves and in some neuroendocrine tumors (54–57). At variance with XLαs and 1A, NESP55 mRNA was only detected in a subset of stromal cell clones. Further studies on the expression of the protein and on the presence of NESP55 transcript in a subset of stromal clones are needed, and raise the interesting issue of the potential neuroendocrine profile of a subset of CFU-Fs. As in previous studies reporting the exclusive maternal expression of NESP55 (16), we detected only one allelic type of NESP55 cDNA in all positive samples. Although the maternal origin of NESP55 transcript could not be directly demonstrated in this study, it was strongly suggested by the observation that in individual clones, NESP55 cDNA showed an opposite allelic origin compared with 1A cDNA, the expression of which has been reported to be restricted to the paternal allele (58). Regardless of their parental origin, the monoallelic expression of NESP55 cDNA and 1A cDNA in our clones clearly demonstrate that the imprinting status of *GNAS* is maintained in bone marrow stromal cells during *ex vivo* expansion. This strongly support the validity of the experimental approach used in this study and provide a positive control for the results obtained with Gsα.

In conclusion, we have shown that there is a high level of clonal heterogeneity in the expression profile of *GNAS* transcripts in bone marrow stromal cells (including skeletal stem/progenitor cells), both in the allelic expression of Gsα and in the portfolio of *GNAS* transcripts. This heterogeneity may play a role in the phenotypic variations of the human skeleton and affect the skeletal phenotype of FD patients.

MATERIALS AND METHODS

Patients

Fresh skeletal samples were obtained from 17 FD patients (Pt 1–17) and 12 normal donors (N 1–12) with informed consent and under institutionally approved protocols for the use of human subjects in research. A diagnosis of FD or MAS was made based on clinical assessment and mutation analysis. A summary of patient data is reported in Table 1.

Bone marrow stromal cell cultures

Bone marrow stromal cell cultures (BMSCs) were established from 17 FD patients and 12 normal donors as previously described (33). Briefly, fresh FD bone/marrow tissues were scraped into nutrient medium consisting of an αMEM (Biofluids, Inc., Rockville, MD, USA), 20% FBS (Life Technologies, Inc., Gaithersburg, MD, USA), 100 U/ml penicillin–100 μg/ml streptomycin (Life Technologies, Inc.) and 1 mM glutamine (Biofluids, Inc.) and pipetted repeatedly to generate single-cell suspensions. Samples were then passed consecutively through needles of decreasing diameter and a 70 μm cell sieve (Falcon Labware, Franklin Lakes, NJ, USA) and then plated in complete nutrient medium. Because it is known that FD lesions are composed of a mixture of normal and mutated stromal cells, cell suspensions from Pts 4, 8, 10 and 13 were plated at $0.007–3.5 \times 10^3$ nucleated cells/cm^2 (clonal density) in order to obtain well-separated stromal cell colonies, each of which represented the progeny of an individual wild-type or *GNAS* R201-mutated CFU-F. Individual colonies were isolated, expanded and genotyped by direct DNA sequencing of relevant PCR products. The cell suspension from another FD patient (Pt 17) was plated at a density of 1×10^5 cells/cm^2 to generate non-clonal cultures in which the presence of *GNAS* R201-mutated cells was confirmed by DNA sequencing. Table 2 lists the different FD cell populations available for study and results of mutation analysis.

Normal BMSCs were established from iliac crest aspirates obtained from 12 normal donors. Briefly, whole bone marrow aspirates, containing 100 U/ml sodium heparin, were mixed with Hank's Balanced Salt Solution, pH 7.2 (Life Technologies, Inc.), containing 30 mM HEPES (Sigma, St. Louis, MO) and 100 U/ml penicillin–100 μg/ml streptomycin. After a brief centrifugation at 4°C, cells were resuspended in fresh medium and single-cell suspensions were generated by passing through a 18 gauge needle and a 70 μm pore-size cell strainer. Cells were plated at a density of 4×10^5 cell/cm^2 to generate non-clonal cultures.

Genomic DNA extraction

Genomic DNA was extracted from fresh skeletal tissues or bone marrow stromal cells, using the Puregene DNA isolation kit according to manufacturer's instruction (Gentra, Minneapolis, MN, USA).

R201 mutation analysis

R201 mutations were determined in 200–500 ng of genomic DNA isolated from tissue and cells (clonal and non-clonal) in a 100 μl PCR reaction by two different methods as reported previously (32). In cases where the majority of the cells were mutated, direct DNA sequencing was used. For the PCR reaction, Perkin Elmer (Foster City, CA, USA) reagents and 2.5 U of Ampli *Taq* Gold polymerase and 1.5 μM each of forward (5′-TGACTATGTGCCGAGCGA-3′) and reverse (5′-CCACG TCAAACATGCTGGTG-3′) primer (GenBank Accession Number: M21142.1, bases 272–289 and 611–592, respectively). Samples were heated to 94°C for 15 min to activate the polymerase, cycled 35 times (94°C for 60 s, 55°C for 30

s and 72°C for 60 s), and terminated for 7 min at 72°C. The PCR products were purified using the Promega Wizard PCR Preps DNA Purification System (Madison, WI, USA). The DNA was then sequenced using an internal (reverse) primer (5′-CCACGTCAAACATGCTGGTG-3′) by using dRhodamine dye-terminator cycle sequencing with Ampli *Taq* and the Perkin Elmer Applied Biosystems 377 automated sequencer.

For cases in which the majority of the cells in culture were not mutant (Pts 5–8), we used a peptide nucleic acid (PNA) whose binding site overlapped the forward oligonucleotide to suppress the amplification of the non-mutant sequence (32). Perkin Elmer reagents and 2.5 U of Ampli *Taq* Gold polymerase were used with 1.5 μM each of forward (5′-GTTTCAGGACCTGCTTCGC-3′) and reverse (5′-GCAA AGCCAAGAGCGTGAG-3′) primer (GenBank Accession Number: M21142.1, bases 420–438 and 746–728, respectively) and 2 μg of PNA (N-terminal 5′-CGCTGCCGTGTC C-terminal-3′). The samples were heated to 94°C for 15 min, cycled 40 times [94°C for 30 s, 68°C for 60 s (to allow the PNA to bind specifically to any non-mutant allele and block the annealing of the forward oligonucleotide), 55°C for 30 s, and 72°C for 60 s], and terminated for 7 min at 72°C. The 340 bp product (direct sequencing) and 325 bp product (PNA) were purified and sequenced as described previously (32).

Amplification of genomic DNA for determination of the ATT/ATC polymorphism in exon 5

A 429 bp fragment of the *GNAS* gene including exon 5 in which an ATT/ATC (Isoleucine-131-Isoleucine) polymorphism has been described and was chosen as target for PCR amplification. The target sequence was amplified in a standard 100 μl PCR reaction using the following primers: forward (PolyS) 5′-ATGAAAGCAGTACTCCTAACTG-3′ and reverse (PolyAS) 5′-TGGATGCTCCTGCCCATGTG-3′ (GenBank Accession Number: M21741.1, bp 12–33, 421–440, respectively) and 2.5 U of *Taq* Gold DNA polymerase enzyme (Perkin Elmer, Norwalk, CT). After a denaturation step at 94°C for 15 min, 34 cycles of amplification were performed at the following temperatures: 94°C, 45 s; 58°C, 45 s; 72°C, 45 s with a final extension time of 10 min at 72°C. After amplification, PCR products were used for DNA sequencing and restriction analysis.

DNA sequencing

For DNA sequencing, PCR samples were purified using the QiAmp PCR purification kit (Qiagen, Valencia, CA, USA) and then, sequenced using dRhodamine dye-terminator cycle sequencing with Ampli *Taq* and the Perkin Elmer Applied Biosystem 377 Automated sequencer.

Fok I restriction analysis

The substitution of C for T in the codon for Isoleucine-131-Isoleucine creates a restriction site for Fok I, which specifically cleaves 13 and 9 nucleotides away from the CATCC recognition site on the sense and anti-sense strands, thereby allowing the identification of homozygous (ATT/ATT, ATC/ATC) or polymorphic (ATT/ATC) patients. Following amplification, 1 mg of PCR products was incubated with 1 U of Fok I (Hoffmann-La Roche Ltd, Basel, Switzerland) for 1 h at 37°C. After digestion, reaction mixtures were run on pre-cast 6% TE acrylamide gels (Invitrogen, Carlsbad, CA) and visualized by staining with ethidium bromide and ultraviolet illumination.

Reverse transcriptase–polymerase chain reaction

Total RNA was extracted from confluent cultures by Trizol (Sigma) according to the manufacturer's instructions. One microgram of total RNA from each sample was digested with 1 microlitre of 10X Deoxyribonuclease I (Fermentas Inc., Hanover, MD, USA) for 10 min at 37°C and total cDNA was synthesized using the Superscript™ Preamplification System with oligo dT according to the manufacturer's instructions (Life Technologies, Inc.). A target sequence of each *GNAS* transcript (Gsα, XLαs, NESP55 and 1A) cDNA was amplified using different forward primers, complementary to the transcript-specific first exons. The forward primers used were as follows: Gsα F 5′-CCATGGGCTGCCTCGGGA ACA-3′ (GenBank Accession Number: BC008855.2, bp 141–161); XLαs F 5′-CGCAGTAAGCTCATCGACAAA-3′ (GenBank Accession Number: NM080425.2, bp 1609–1629); NESP55 F 5′-AGCCCGAGGACAAAGATCCA-3′ (GenBank Accession Number: 016592.2, bp 951–970); 1A F 5′-TGGAGCGA GCCCCTGTC-3′ (GenBank Accession Number: X56009.1, bp 133–149). Two reverse primers were used: common R 5′-CCTTGGCATGCTCATAGAATTC-3′ (GenBank Accession Number: BC008855.2, bp 599–578) for Gsα, XLαs and NESP55 cDNAs amplification and common R2 5′-CTCGTTGGAGCGTTCGT -3′ (GenBank Accession Number: X56009.1, bp 580–564) for 1A cDNA amplification. The target sequences were amplified in a standard PCR reaction using 2.5 U of the *Taq* Gold DNA polymerase (Perkin Elmer, Norwalk, CT, USA). After a denaturation step at 94°C for 15 min, amplification was performed as follows: Gsα, 35 cycles at 94°C (45 s), 62°C (45 s), 72°C (45 s); XLαs, 37 cycles at 94°C (30 s), 55°C (30 s), 72°C (45 s); NESP55, 35 cycles at 94°C (45 s), 58°C (45 s), 72°C (45 s) and 1A, 30 cycles at 94°C (30 s), 56°C (30 s), 72°C (60 s). The final extension for each primer was at 72°C for 7 min. After amplification, samples were run on pre-cast 6% TE acrylamide gels (Invitrogen). PCR products were verified by direct sequencing of the first two exons and then used for Fok I restriction analysis as described above.

Cloning, digestion and sequencing of GSα RT–PCR products

The Gsα cDNAs amplified from two normal and two mutated FD clones were purified by Qiagen kit and cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen) according to the manufacturer's instructions. Briefly, a TOPO TA cloning reaction was prepared for each RT–PCR product and used to transform chemically competent *Escherichia coli* (Top Ten) cells. Cells were grown on medium selective for only those bacteria containing inserted sequence, and 12 clones were selected for each sample. The plasmid was purified using the Qiagen kit and the sequence of the insert was analyzed by Fok I digestion and DNA sequencing as described above.

Densitometric analysis

After digestion with Fok I, equal amounts (determined spectroscopically) of PCR products were separated on 2% agarose gels, visualized by staining with ethidium bromide and ultraviolet illumination and evaluated by an imaging densitometer, Gel Doc 2000 (BioRad, Hercules, CA, USA). The total amount of ATT-cDNA and ATC-cDNA in each sample was calculated as the sum of the intensity of the corresponding bands and expressed as percentage of the total PCR product loaded in each well.

q-PCR analysis

q-PCR analysis for Gsα was performed using the allelic discrimination method (33). Two FAM-labeled probes, specifically recognizing the ATT or ATC sequence in exon 5, and a single set of primers were synthesized. The probes and primers sequences were as follows: 5′-FAM-TGGACTACA TCCTGAGTGTGAT-TAMRA-3′ (537T, ATC probe, GenBank Accession Number: BC008855, bp 528–549 in exon 5); 5′-FAM-TGGACTACATTCTGAGTGTGAT-TAMRA-3prime; (537T1, ATT probe, GenBank Accession Number: BC008855, bp 528–549 in exon 5); 5′- GAATTCGGGA GGAAGTCAAA-3′ (513F, forward primer, GenBank Accession Number: BC008855, bp 583–563 in exon 6–5); 5′-CCAACCCCGAGAACCAGTT-3′ (529R, reverse primer, GenBank Accession Number: BC008855, bp 504–522 in exon 5). Since all the different *GNAS* transcripts share the same coding region starting at exon 2, including the ATT/ATC polymorphic site in exon 5, quantitative analysis was performed on target Gsα cDNA, previously amplified by standard RT–PCR (which cannot distinguish between the two alleles) as reported above, and the relative quantification of the two allelic transcripts, as identified by the FAM-labeled probes, was based on the standard curve method.

Two standard curves were generated, one for the ATT and one for the ATC Gsα transcript, as follows. The target Gsα cDNA was amplified from two homozygous patients (Normal donor 1, ATT/ATT; Normal donor 2, ATC/ATC) and then decreasing amounts of ATT–Gsα cDNA were mixed with increasing amount of ATC–Gsα cDNA reaching a constant final cDNA concentration of 0.02 ng. Each ATT/ATC cDNA mixture was then used as a template for two q-PCRs, one with the ATT-specific FAM probe and one with the ATC probe in order to obtain the Ct values corresponding to the different concentration of ATT and ATC cDNA fragments. Reactions were carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystem, Foster City, CA) using a 10^{-7} dilution of RT–PCR products as templates, 100 nM of each probe, 90 nM of each primer and *Taq*-Man Universal PCR Master Mix (Applied Biosystems). Sample analysis was carried out in the same conditions used to generate the standard curves. Two separate reactions were performed on the Gsα cDNA amplified from each patient, one with the ATT specific FAM probe and one with the ATC probe. The amounts of ATT–cDNA and ATC–cDNA were then assessed by comparison between the Ct value of each reaction and the Ct values reported in the standard curves.

Quantitative analysis of NESP55 was performed on the target cDNA (amplified by standard RT–PCR as described above) by applying the same FAM-labeled probes and primer set used for Gsα (recognizing the exon 5 polymorphic site shared by all *GNAS* transcripts). Relative quantification of allelic transcripts was performed by standard curves as reported for Gsα.

ACKNOWLEDGEMENTS

This study was supported by grants from the MIUR to M.R., by the DIR, NIDCR of the IRP, NIH to N.C., K.H., M.T.C. and P.G.R., and by Telethon, Grant #GGP04263 to P.B.

Conflict of Interest statement. None declared.

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